IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Nicholas Lawrence Abbott
Seung-Ryeol Kim

Title: BIOCHEMICAL BLOCKING LAYER
FOR LIQUID CRYSTAL ASSAY

Appl. No.: 10/625,869

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DECLARATION UNDER 37 C.F.R. § 1.131

Commissioner for Patents and Trademarks
P.O. Box 1450
Alexandria, VA 22313-1450

I, Nicholas L. Abbott, state and declare that:

1. I am an inventor of at least Claims 1-20 of U.S. Patent Application No. 10/625,869, entitled "Biochemical Blocking Layer For Liquid Crystal Assay."

2. I am also an inventor of U.S. Application No. 10/934,023 and of U.S. Patent No. 6,284,197.

3. Exhibit A is a copy of a paper my research group published in *Analytical Chemistry*. (*Anal. Chem.* **2000**, 72, 4646-4653).

4. Figure 3 of Exhibit A demonstrates that rubbing the surface of a biochemical blocking layer of BSA supported on an underlying substrate imparts structural features to the

biochemical blocking layer that drive a uniform alignment of a liquid crystal on the biochemical blocking layer and further demonstrates that these structural features are not present on a biochemical blocking layer of BSA that has not been rubbed.

5. Figure 6 of Exhibit A demonstrates that rubbing the surface of a biochemical blocking layer of BSA supported on an underlying substrate imparts structural features to the biochemical blocking layer that drive a uniform alignment of a liquid crystal on the biochemical blocking layer and further demonstrates that these structural features are preserved following the exposure of the rubbed surface of the blocking layer to non-specific binding proteins in solution. Figure 5 of Exhibit A demonstrates that, if a surface other than a biochemical blocking layer is rubbed, the exposure of that rubbed surface to non-specific binding proteins in solution results in the non-specific binding of proteins to the surface, which masks the structural features imparted to the rubbed surface by the rubbing.

6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: October 18, 2006

By: _____
Nicholas L. Abbott

Anal. Chem. **2000**, *72*, 4646−4653

Exhibit A

Orientations of Liquid Crystals on Mechanically Rubbed Films of Bovine Serum Albumin: A Possible Substrate for Biomolecular Assays Based on Liquid Crystals

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We report the uniform planar anchoring of thermotropic liquid crystals on films of bovine serum albumin (BSA) covalently immobilized on the surface of glass microscope slides and mechanically rubbed using a cloth. The azimuthal orientations of the liquid crystals were measured to be parallel to the direction of rubbing. Following immersion and removal of these rubbed films of BSA from aqueous solutions containing either BSA, fibrinogen, lysozyme, anti-FITC immunoglobulin G (IgG), or anti-streptavidin IgG, we measured liquid crystals placed onto these surfaces to largely retain their uniform alignment. In contrast, following immersion of a rubbed film of BSA into an aqueous solution of anti-BSA IgG, we observed liquid crystals on these surfaces to assume nonuniform orientations. We conclude that specific binding of anti-BSA IgG to the film of rubbed BSA erased anisotropy induced within the film of BSA by rubbing. This result suggests that the spatial scale of anisotropy within the rubbed film of BSA is comparable to or smaller than the size of the IgG molecule. Because the anisotropy within a rubbed film of a protein can be erased by specific binding of a second protein, we believe these types of substrates (rubbed films of proteins) have the potential to be useful in a variety of label-free biomolecular assays where specific binding of a target species to its ligand can be imaged through observation of the optical appearance of liquid crystal placed onto the surface.

We have recently reported that liquid crystals can be used to amplify and transduce the receptor-mediated binding of proteins at surfaces into optical outputs.[1] By hosting ligands for specific proteins on appropriately designed surfaces, we have demonstrated that the binding of target proteins to the ligands can trigger changes in the orientations of 2−20-μm-thick films of supported liquid crystals. These changes in orientation of the liquid crystals can be readily observed by using polarized light. This approach (i) permits imaging of region-specific binding of proteins to surfaces with micrometer resolution, (ii) can be performed rapidly, and (iii) does not require the use of enzymatic or fluorescent labels. Past studies have also shown that visual inspection of the optical appearance of the liquid crystals can be used to detect threshold amounts of proteins whereas gray scale analyses of the optical appearance can yield quantitatively measures of the amount of bound protein.[2]

Past studies of the use of liquid crystals to amplify biomolecular interactions on surfaces have exploited the nanometer-scale structure of self-assembled monolayers (SAMs) of ω-functionalized alkanethiols formed on obliquely deposited films of gold.[1-9] Although the self-assembly of organosulfur compounds on the surfaces of films of gold permits a high level of control over the structure of the surface, this approach requires access to an ultrahigh-vacuum chamber for deposition of the gold films. This requirement is a potential barrier to the widespread use of the principle in biochemical laboratories. In this paper, we describe a procedure that permits the preparation of substrates for liquid crystal-based biomolecular assays that does not require access to apparatus beyond that commonly found in wet chemical laboratories. The approach we report is based on the formation of films of protein covalently attached to the surface of glass microscope slides and the rubbing of these films by using a modified chart recorder. Covalent immobilization of the protein leads to mechanically robust protein films,[10] and the process of rubbing of the films leads to an anisotropic surface structure useful for liquid crystal assays.[11-13] Here we demonstrate this method of preparation of protein films to lead to surfaces that possess four essential properties required for biomolecular assays based on liquid crystals. First, the rubbed films of protein uniformly orient liquid crystals. Second, the rubbed substrates resist the

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nonspecific adsorption of proteins from solution. Third, the rubbed proteins bind specifically targeted biomolecules in solution. Fourth, the binding of the targeted molecules to the rubbed protein films changes the orientation of liquid crystals on these surfaces.

Here we report use of rubbed films of bovine serum albumin (BSA) for several reasons. First, films of BSA are commonly used to block the nonspecific adsorption of biomolecules in biological assays.[14-19] Nonspecific adsorption of proteins and other biomolecles is detrimental to both the sensitivity and specificity of assays. Second, a past study has reported alignment of liquid crystal on a film of BSA deposited onto a substrate by pulling the substrate through the surface of an aqueous solution containing BSA.[20] Third, past reports have described the use of rubbed proteins and polypeptides for alignment of liquid crystals.[21-23] These past studies did not, however, determine whether rubbed films of protein resist nonspecific adsorption of biomolecules, nor did they determine whether specific binding of biomolecular species to rubbed films of proteins will lead to measurable changes in the alignment of the liquid crystals. The work reported in this paper addresses these principal issues.

EXPERIMENTAL SECTION

Materials. The glass microscope slides were Fisher's Finest, premium grade obtained from Fisher Scientific (Pittsburgh, PA). Polished silicon (100) wafers were purchased from Silicon Sense (Nashua, NH). Octadecyltrichlorosilane (OTS) and (3-aminopropyl)triethoxysilane (APES) were purchased from Gelest (Tullytown, PA). Solutions for silanization of glass slides were prepared in anhydrous toluene (Aldrich, Milwaukee, WI) when using OTS and in 10 mM sodium acetate−acetic acid buffer (pH 5.0) when using APES. Disuccinimidyl suberate (DSS) was obtained from Pierce (Rockford, IL). BSA (IgG-free, lyophilized powder), anti-BSA IgG (polyclonal, developed in rabbit), anti-streptavidin IgG (polyclonal, developed in rabbit), anti-FITC IgG (monoclonal, clone FL-D6, mouse ascites fluid), fibrinogen (fraction I, type III from human plasma), and lysozyme (EC 3.2.1.17, grade III, from chicken egg white) were obtained from Sigma (St. Louis, MO) and used as received. All proteins used in this study were dissolved in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4) solution at pH 7.2. All aqueous solutions were prepared with deionized water having a resistivity greater than 18.2 MΩ·cm (Milli-Q^{plus}, Millipore, Bedford, MA). The nematic liquid crystal of 4-cyano-4′-pentylbiphenyl (5CB), manufactured by BDH, was purchased from EM Industries (Hawthorne, NY).

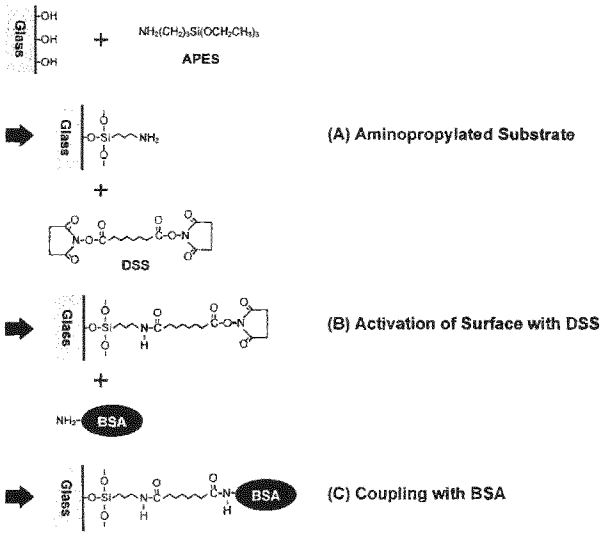

Figure 1. Experimental procedure used to covalently immobilize BSA onto the surfaces of glass microscope slides: (A) reaction of the ethoxy groups of APES with hydroxyl groups presented at the surface of the glass slide to form a siloxane bond; (B) activation of the aminopropylated substrate by reaction with the homobifunctional cross-linker (DSS); (C) reaction of the free succinimide ester groups of the activated substrate with the amine residues of BSA.

Cleaning of Substrates. The glass microscope slides and silicon wafers were cleaned by using "piranha solution" (70% H_2SO_4/30% H_2O_2, *Caution: "piranha solution" reacts violently with organic materials and should be handled with extreme caution; do not store the solution in closed containers*) for 1 h at 80 °C. After removal from the cleaning solution, the substrates were rinsed with copious amounts of deionized water and dried under a stream of nitrogen. The cleaned substrates were stored in an oven (120 °C) for at least 3 h before use.

Physically Adsorbed Films of BSA. Our initial experiments were based on films of BSA physically adsorbed to both hydrophobic and hydrophilic substrates. We used clean silicon wafers with native oxide films as hydrophilic substrates. Hydrophobic substrates were prepared by immersing silicon wafers overnight in 3% OTS dissolved in anhydrous toluene. To reduce the extent of hydrolysis of the OTS by ambient water, we performed the silanization reaction within a nitrogen glovebox (model CC-40, Vacuum Atmospheres Co., Hawthorne, CA). The silanized substrates were rinsed with toluene and dried at 120 °C for at least 3 h. Physically adsorbed layers of BSA were prepared by immersing the substrates overnight in solutions of 1.0 mg/mL BSA in PBS (pH 7.2).

Covalently Immobilized Films of BSA. The experimental procedure used to covalently immobilize BSA on the surfaces of glass microscope slides (or silicon wafers) is shown schematically in Figure 1. Clean substrates were aminopropylated by reaction for 3 h at 80 °C with 10% APES in a sodium acetate−acetic acid buffer (10 mM, pH 5.0). The aminopropylated substrates were rinsed with deionized water and dried at 120 °C for at least 3 h before they were activated with a succinimide ester cross-linker (DSS) to promote the coupling of the BSA to the surface by amide

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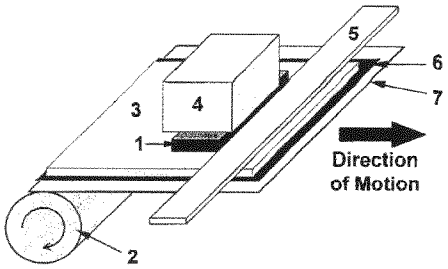

1. substrate
2. modified strip chart recorder
3. velvet-type polyester cloth
4. aluminum block
5. barrier
6. double-sided tape
7. chart paper

Figure 2. Schematic illustration of the experimental apparatus used to rub films of covalently immobilized BSA. Each film of BSA was immobilized on a substrate (see Figure 1) and placed onto the surface of chart paper on a modified chart recorder. A velvet-type cloth was attached to the base of an aluminum block and placed onto the surface of the film of BSA. The motion of the substrate under the aluminum block caused the film of BSA to be rubbed by the cloth.

bond formation. The aminopropylated substrates were immersed in anhydrous methanol, and then a 50 mM DSS stock solution in anhydrous DMSO was added to the methanol solution (final concentration of DSS was 1.0 mM). The substrates were immersed in the stirred solution for 1 h, washed with methanol and deionized water, and then immediately coupled to amine groups of BSA. The BSA coupling was achieved by immersing the DSS-activated substrates in a 1.0 mg/mL BSA solution in PBS buffer (pH 7.2) for overnight.

Rubbed Films of BSA. Rubbed films of BSA were prepared by sliding a velvet-type cloth (90% polyester/10% Spandex, Logantex Inc., New York) across BSA-coated substrates using a strip chart recorder (model SR-255 A/B, Heath Co.) that was modified as shown in Figure 2. The cloth was attached to the chart paper using double-sided tape, and the BSA-coated substrate was placed (face down) on the cloth. The film of BSA was rubbed by the movement of the cloth beneath the substrate. Each film of BSA was rubbed for 1 min at a speed of ~4.2 mm/s. A pressure of 10^3 Pa was applied to the surface of the film of BSA by placement of an aluminum block weighing ~200 g onto the substrate (2 cm × 7 cm).

Rubbed Substrates Not Supporting Films of BSA. For purposes of comparison to rubbed films of BSA, we also rubbed glass slides that were not coated by BSA and prepared glass slides onto which we shear-deposited a film of poly(tetrafluoroethylene) (PTFE). The rubbed glass slides were prepared by mechanical rubbing of bare glass slides under the same conditions used to prepare the rubbed films of BSA. The shear-deposited PTFE films were prepared by sliding a flat PTFE block across glass slides that were heated to a temperature of ~100 °C. Past studies have demonstrated that films of PTFE deposited in this manner will orient liquid crystals.[24] The applied pressure and speed were controlled and were ~10^4 Pa and ~0.5 mm/s, respectively.

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Protein Adsorption. We investigated the nonspecific and specific binding of proteins to rubbed films of covalently immobilized BSA by incubation of these substrates in PBS solutions of proteins (pH 7.2) for 2 h. We used solutions of 100 nM (0.01 mg/mL) IgG (anti-BSA IgG, anti-streptavidin IgG, and anti-FITC IgG), 10 mg/mL BSA, and 0.2 mg/mL fibrinogen and lysozyme. The substrates were rinsed with deionized water and dried under a stream of nitrogen upon removal from the protein solutions.

Ellipsometric Thickness. All ellipsometric measurements were performed using silicon wafers covered with films of native oxide as the substrate (not glass microscope slides). Ellipsometric thicknesses were measured at three points on each sample by using a Rudolph Auto EL ellipsometer (Flanders, NJ) at a wavelength of 6320 Å and an angle of incidence of 70°. We compared the use of multilayer (Si/SiO_2/OTS) and two-layer models (substrate/OTS) of OTS SAMs and found that the ellipsometric thicknesses of the OTS SAMs were accurately predicted by the two-layer model (20 ± 1 Å). We therefore modeled our system using the two-layer model. We interpreted the ellipsometric thicknesses of the adsorbed protein layers by assuming the refractive index to be 1.46 for all the organic layers (protein/BSA/silane).[27]

Optical Cells. We observed the alignment of nematic liquid crystal (5CB) on the surfaces of the rubbed films by assembling the films into optical cells.[3] Optical cells were fabricated by pairing two glass slides, each of which supported a rubbed film. The rubbed films were aligned (facing each other) such that the directions of rubbing were parallel within the cell. The rubbed films were kept apart by inserting thin polyester film (~10-μm thickness of Mylar, DuPont Films, Wilmington, DE) between the surfaces of the rubbed films. The cells were held together by using "bulldog" clips placed along the edge of the glass microscopic slides. The cells were heated to ~40 °C by placing them on a hot plate. We also used a hot air gun to warm the air around the cells to ~40 °C. The 5CB was heated into its isotropic phase (~35 °C) within a glass syringe. A drop of 5CB was placed onto the edge of each cell on the hot plate. The 5CB was drawn into the cavity between the two rubbed surfaces by capillary force. Once filled with 5CB, the cell was removed from the hot plate and cooled in air to room temperature. Upon cooling, the isotropic phase of 5CB transformed to the nematic state.

Polarized Light Microscopy. A polarized light microscope (BX60, Olympus, Tokyo, Japan) was used to observe the optical textures formed by light transmitted through the optical cells filled with nematic 5CB. All images were obtained using a 20× objective lens with a 550-μm field of view between crossed polarizers. The azimuthal orientations of the liquid crystals were determined by

(25) When illuminated using white light and crossed polarizers, a material possessing birefringence ($\Delta n = n_e - n_o$, where n_e is the extraordinary refractive index and n_o is the ordinary refractive index) causes, in general, the formation of interference colors. These colors result from unequal transmission by the analyzer of different wavelengths of light within white light. The extent to which the analyzer transmits (or absorbs) each wavelength depends on the retardation (Δnd) of light during its passage through the birefringent material of thickness d. The resulting interference colors are divided into "orders" according to whether they result from retardations of 0–550 (first-order colors), 550–1100 (second-order), 1100–1650 nm (third-order), and so on.

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changes in the interference colors upon insertion of a quarter-wave plate (Normarski prism, 147.3 nm) into the optical path.[25] These measurements were performed by placing the optical cells into the microscope with the rubbing direction parallel to the slow axis of a quarter-wave plate. The slow axis of the liquid crystal was determined by observing the shift in interference color. Upon insertion of the quarter-wave plate, the interference color shifted toward higher retardation in the Michel–Lévy chart[26] when the slow axis of the liquid crystal and the quarter-wave plate coincided.

Out-of-Plane Orientations of 5CB. A home-built optical apparatus was used to measure the out-of-plane orientation (tilt angle) of 5CB within the optical cells. The optical cells were placed between crossed polarizers, illuminated at normal incidence using a polarized He–Ne laser (model 05-LHP-141, Melles Griot, Carlsbad, CA), and then rotated from −20° to +20° with respect to the normal. A plot of the intensity of light transmitted through the cell against the angle of incidence was used to estimate the tilt of the optical axis of the liquid crystals from the surface of the cell.[28]

Transmittance of Optical Cells. The intensity of light transmitted through each optical cell, i, was recorded during rotation of the sample between crossed polarizers. The background intensity ($i_{background}$) of light transmitted through crossed polarizers and the maximum intensity ($i_{parallel}$) of light transmitted through parallel polarizers were also recorded using an empty optical cell (without 5CB). The values of intensity reported here are corrected for the background intensity of light that passed through the crossed polarizers and are normalized by the intensity of light measured to pass between parallel polarizers (both empty cells). That is, the corrected and normalized fractional transmittance, I, is given by

$$I = (i - i_{background})/i_{parallel} \quad (1)$$

All intensities of light transmitted through the optical cells were measured using a photodiode (silicon photodiode FDS100, Thorlabs, Inc., Newton, NJ).

RESULTS AND DISCUSSION

Rubbed Films of Physically Adsorbed BSA. It is well known that BSA will adsorb to hydrophobic substrates immersed in aqueous solutions of BSA.[29,30] Films prepared in this manner are widely used as blocking layers in biological assays such as enzyme-linked immunosorbent assays (ELISAs).[31] Films of BSA are also known to form on the surfaces of hydrophilic substrates.[32] At the outset of our experiments, therefore, we investigated the rubbing of films of BSA physically adsorbed to clean, hydrophilic silicon wafers and hydrophobic, OTS-treated silicon wafers. Our measurements of ellipsometric thicknesses of these films of BSA indicated that over 50% of the physically adsorbed BSA was removed by rubbing, independent of the hydrophobicity of the surface on which the protein was physically adsorbed (Table 1). As described below, we also found that rubbed films of physically adsorbed BSA did not reliably resist nonspecific adsorption of proteins. These results, when combined with past reports in the literature that have described physisorbed films of BSA to be susceptible to removal by rinsing or displacement by other proteins,[33–35] caused us to explore ways to prepare mechanically robust films of BSA.

Table 1. Influence of Mechanical Rubbing on the Ellipsometric Thicknesses of Adsorbed Films of BSA[a]

substrate	ellipsometric thickness of BSA layer, Å: before rubbing	after rubbing
SiO_2/Si	11 ± 1	5 ± 1
OTS on SiO_2/Si[b]	15 ± 1	3 ± 1
APES on SiO_2/Si[c]	17 ± 2	15 ± 3

[a] BSA layers were prepared by immersing the substrates in solutions of 1.0 mg/mL BSA in PBS buffer (pH 7.2) for overnight. [b] Measured ellipsometric thicknesses of SAMs of OTS were 20 ± 1 Å. [c] Measured ellipsometric thicknesses of SAMs of APES were 11 ± 1 Å.

We prepared mechanically robust films of BSA by using the homobifunctional cross-linker DSS to covalently attach BSA to aminopropylated silicon wafers (Figure 1).[10] The DSS cross-linker reacts with one or more of the 59 lysine ϵ-amino groups of BSA. It is thought that approximately 30–35 of these lysine groups are available for the coupling reaction.[36] In contrast to the physically adsorbed films of BSA, we found that rubbing of covalently immobilized films of BSA did not result in substantial changes in the ellipsometric thicknesses of the BSA films (Table 1). We measured the ellipsometric thicknesses of covalently immobilized films of BSA to decrease in thickness by less than 3 Å upon rubbing.

Orientation of 5CB on Rubbed Films of Covalently Immobilized BSA. Figure 3A shows the optical appearance (crossed polarizers) of a film of nematic 5CB sandwiched between two glass slides presenting covalently immobilized films of BSA (not rubbed). When 5CB was anchored on films of BSA that were not rubbed, we observed the optical appearance of the liquid crystals to be complex and nonuniform. The nonuniform appearance of the liquid crystal indicates that the nematic phase of 5CB is anchored without a preferred azimuthal orientation on these films of BSA. In contrast, when 5CB was sandwiched between surfaces presenting rubbed films of BSA, we measured the optical appearance of 5CB to be uniform and featureless (Figure 3B). This result indicates that the liquid crystals are uniformly oriented on the surface of the rubbed film of BSA. The azimuthal orientation of the liquid crystal on the rubbed BSA was determined by the change in color of the liquid crystal upon insertion of a quarter-wave plate into the optical path (see details in Experimental Section). Insertion of the quarter-wave plate into the optical path resulted in a change in color indicative of an increase in retardation (from Figure 3C to D). This result demonstrates that

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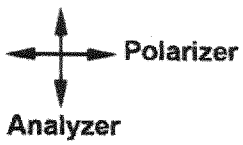

Figure 3. Optical textures of nematic 5CB (crossed polarizers) sandwiched between glass microscope slides supporting films of BSA. The optical textures were obtained (A) before and (B) after rubbing of the films of BSA. The direction of rubbing was parallel to the polarizer. The optical texture shown in (C) was obtained after rotation of the cell in (B) by 45°. The image in (D) was obtained using the sample shown in (C) following insertion of a quarter-wave plate into the optical path. The white arrows indicates the rubbing direction, and the red arrows indicate the optical axis of quarter-wave plate (Normarski prism, 147.3 nm) with the high refractive index (i.e., slow axis). The horizontal dimension of each sample shown is 550 μm.

liquid crystals of 5CB align on these surfaces in a direction that is parallel to the direction of rubbing of the BSA film. We also measured the out-of-plane orientation (tilt angle) of 5CB supported on rubbed films of BSA. By using the crystal rotation apparatus (see details in Experimental Section), we measured the tilt angle of the optical axis of 5CB from the plane containing the rubbed BSA layer to be 1.5 ± 0.5°.

When rotated between crossed polarizers, the optical appearance of 5CB anchored between the rubbed films of BSA was observed to modulate between dark (Figure 3B) and light (Figure 3C). The liquid crystals appear dark when the optical axis of the nematic phase aligns with either the polarizer or the analyzer. We used measurements of the modulation of the intensity of light transmitted through the optical cell during its rotation to quantify the uniformity of alignment of the liquid crystals. As detailed in the Experimental Section, we quantified these measurements in terms of a fractional transmittance. We measured a strong modulation in the fractional transmittance of light when the liquid crystal was supported on the rubbed film (Figure 4). In contrast, little modulation, if any, in the fractional transmittance was measured during rotation of the liquid crystal supported on the film of BSA that had not been rubbed (Figure 4).

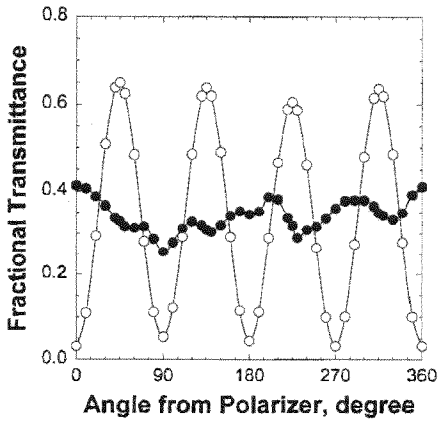

Figure 4. Fraction of light transmitted through nematic 5CB anchored on the surface of rubbed films of BSA as a function of the angle between the direction of rubbing and the polarizer (open circles). For comparison, the fraction of light transmitted through a film of 5CB supported on a BSA film prior to rubbing is shown (filled circles). All fractional transmittances were obtained using one field of view of the liquid crystal.

These measurements, when combined, lead us to conclude that rubbed films of chemically immobilized BSA induce "uniform" and "planar" anchoring of nematic phases of 5CB in a direction that is parallel to the rubbing direction.

Orientations of 5CB on Rubbed Glass and PTFE Following Their Immersion into Aqueous Solutions of Proteins. Whereas many surfaces that have been rubbed with a cloth will orient liquid crystals in a uniform manner, we suspected that the level of nonspecific adsorption of proteins on most of these surfaces would be sufficiently high that the uniform orientation of the liquid crystals would be erased by the adsorbed proteins. To test this prediction, we investigated the influence of nonspecific adsorption of proteins on rubbed hydrophilic glass slides (not supporting a deliberately adsorbed film of polymer) and hydrophobic PTFE films that were shear-deposited onto the surface of glass slides.[24] As shown in Figure 5A and C, we observed uniform alignment of 5CB on the rubbed glass slides and the shear-deposited PTFE films prior to immersion into aqueous solutions of BSA. However, after immersion and withdrawal of both types of substrates from aqueous solution containing 0.1 mg/mL BSA, we measured the alignment of the liquid crystals to be nonuniform on both surfaces (Figure 5B and D). This result indicates that the level of nonspecific adsorption of BSA on these rubbed surfaces was sufficient to mask the anisotropy induced in the surface by the process of rubbing. We draw two conclusions from

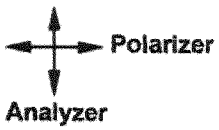

Figure 5. Optical textures (crossed polarizers) of 5CB sandwiched between rubbed glass slides (A, B) and between PTFE-rubbed glass slides (C, D). The optical textures were obtained before (A, C) and after immersion in 0.1 mg/ml BSA solution in PBS buffer (pH 7.2) for 2 h (B, D). The direction of rubbing was parallel to the polarizer. The white arrow indicates the rubbing direction. The horizontal dimension of each sample shown is 1.1 mm.

these results. First, the extent of nonspecific adsorption of protein on these surfaces will prevent their use as substrates for biomolecular assays that are based on liquid crystals because the nonspecific adsorption will mask the effects of specific binding of proteins to the surfaces. Second, this result suggests that a rubbed surface that does resist nonspecific adsorption may well be useful for biomolecular assays based on liquid crystals because the adsorption of proteins to these surfaces can erase the anisotropic structure within the rubbed surface that orients liquid crystals uniformly.

Orientations of 5CB on Rubbed Films of BSA Following Their Immersion into Solutions Containing Nonspecific Proteins. Whereas the rubbed glass microscope slides and films of PTFE described above do not resist nonspecific adsorption of BSA at levels that lead to uniform alignment of liquid crystals, we next investigated whether the rubbed films of BSA, in contrast, would retain their ability to resist protein adsorption at levels that would yield uniform alignment of 5CB following immersion in protein solutions. Figure 6A shows the optical texture of 5CB supported on a rubbed film of covalently immobilized BSA after immersion of the rubbed film in an aqueous solution containing 10 mg/mL of BSA. When compared with the appearance of the liquid crystal on the rubbed film of BSA shown in Figure 3B, the optical appearance of the liquid crystal is changed little by immersion of the rubbed film of BSA into the solution of BSA. This result contrasts to the optical appearance of the liquid crystals on the rubbed films of PTFE and glass following immersion in the aqueous solution of BSA (Figure 5B and D). We measured the ellipsometric thicknesses of films of BSA with and without rubbing after immersion in the solution of BSA (Table 2). Inspection of Table 2 reveals that a covalently immobilized film of BSA (not rubbed) does not adsorb a measurable amount of BSA when immersed and withdrawn from an aqueous solution containing BSA. In contrast, when rubbed, the covalently immobilized layer of BSA is measured to adsorb ~14 Å of BSA. We conclude, therefore, that the level of nonspecific adsorption of BSA is greater on the rubbed film of BSA as compared to the film of BSA that was not rubbed. The level of additional adsorption of BSA on the rubbed film of BSA, however, was insufficient to disrupt the uniform anchoring of the liquid crystals. As shown below, this result contrasts with the effects of specific binding of anti-BSA IgG to rubbed films of BSA (Figure 6D). In this latter case, we observed the specific binding of anti-BSA IgG to trigger the nonuniform anchoring of liquid crystals on the rubbed film of BSA (see below).

We also investigated the optical appearance of 5CB anchored on rubbed films of BSA that were immersed and withdrawn from aqueous solutions containing fibrinogen and lysozyme. Whereas immersion of a rubbed film of BSA into an aqueous solution of lysozyme resulted in a uniform orientation of the liquid crystal, a number of defects (loop disclinations) appeared in the optical textures of liquid crystals supported on films of rubbed BSA immersed into fibrinogen. Although defects were evident in the optical appearance of the liquid crystal supported on the film immersed into the solution of fibrinogen, the bulk of the liquid crystals remains uniformly oriented. Below we quantify the level of nonuniformity induced by adsorption of fibrinogen (by measurement of fractional transmittance) and show it is clearly distinguishable from the appearance of liquid crystal in cases where anti-BSA IgG binds specifically to the rubbed film of BSA (Figure 7). Whereas the optical appearance of the liquid crystals supported on the rubbed BSA previously immersed into aqueous solutions of BSA (Figure 6A) and fibrinogen (Figure 6C) differs from one another, our measurements of the ellipsometric thickness of nonspecifically adsorbed BSA and fibrinogen reveal very similar levels of adsorption (Table 2). This result demonstrates that liquid crystals can distinguish between types of adsorbed protein layers that are indistinguishable when characterized by ellipsometric methods. We also note that nonspecific adsorption of fibrinogen (~15 Å) was measured on films of BSA, independent of whether the film was rubbed.

We also measured the tilt angles of 5CB after nonspecific adsorption of BSA and fibrinogen on the rubbed films of BSA. We measured the tilt angles to be 3.8 ± 0.8° and 3.5 ± 0.5° for

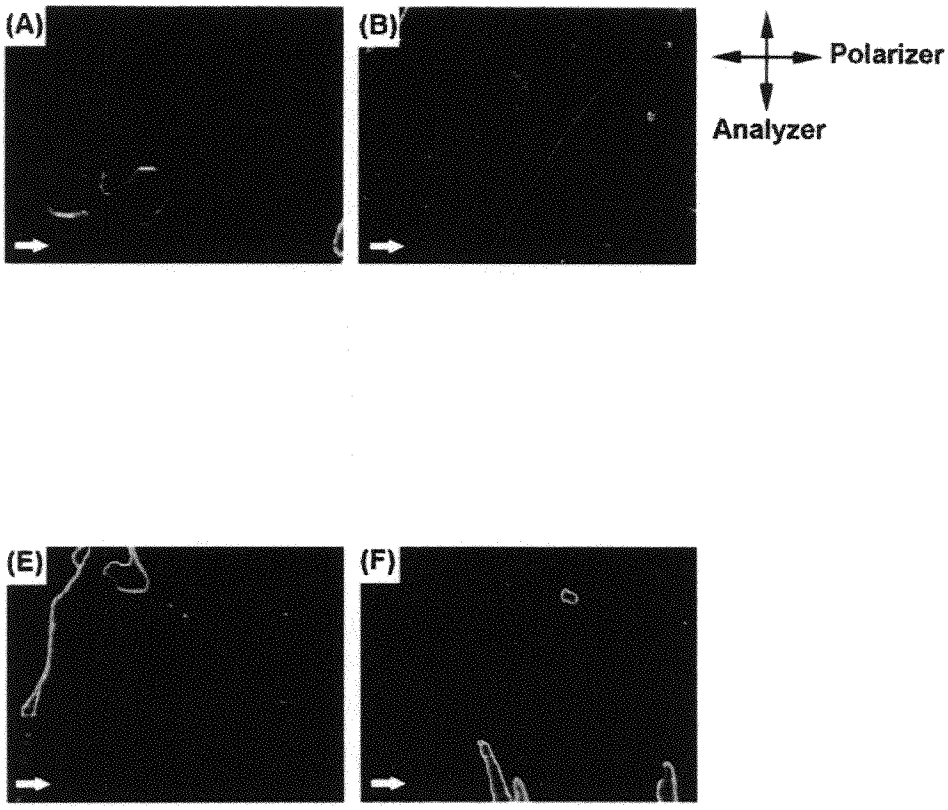

Figure 6. Optical images (crossed polarizers) of 5CB sandwiched between rubbed films of BSA after incubation of the films in aqueous solutions of proteins: (A) 10 mg/mL BSA, (B) 0.2 mg/mL lysozyme, and (C) 0.2 mg/mL fibrinogen solutions in PBS for 2 h, and 100 nM IgG solutions in PBS of (D) anti-BSA IgG, (E) anti-FITC IgG, and (F) anti-streptavidin IgG for 2 h. The white arrow indicates the rubbing direction. The optical images were obtained when the samples were placed in the parallel direction with the polarizer. The horizontal dimension of each sample shown is 550 μm.

Table 2. Ellipsometric Thicknesses of Proteins Adsorbed onto Covalently Immobilized Films of BSA

	increase in thickness due to protein adsorption, Å	
protein (concentration)	BSA film not rubbed	rubbed BSA film
BSA (10 mg/mL)	1 ± 4	14 ± 3
fibrinogen (0.2 mg/mL)	17 ± 4	14 ± 7
lysozyme (0.2 mg/mL)	10 ± 4	12 ± 4
anti-BSA IgG (100 nM)[a]	46 ± 4	38 ± 4
anti-FITC IgG (100 nM)	5 ± 1	7 ± 1
anti-streptavidin IgG (100 nM)	2 ± 1	2 ± 1

[a] 100 nM of anti-BSA IgG corresponds to 0.015 mg/mL.

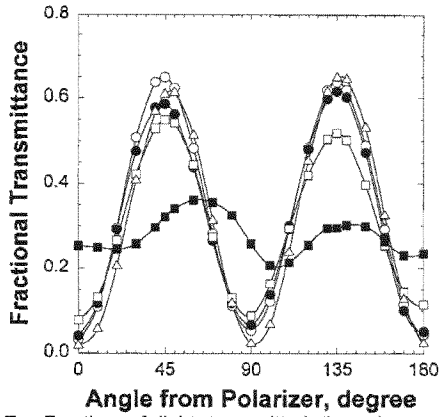

Figure 7. Fraction of light transmitted through nematic 5CB anchored on the surfaces of rubbed films of BSA after immersion of the films into various protein solutions. The transmittance is shown as a function of the angle between the direction of rubbing and polarizer (crossed polarizers). The rubbed films of immobilized BSA were incubated in 10 mg/mL BSA (filled circles) and 0.2 mg/mL fibrinogen (open squares) solutions in PBS buffer for 2 h or in 100 nM anti-BSA IgG (filled squares) and 100 nM anti-FITC IgG (open triangles) solutions in PBS buffer for 2 h. For reference, the fractional transmittance of 5CB on a rubbed film of immobilized BSA prior to immersion into a protein solution was measured (open circles). Each measurement is based on one field of view of the liquid crystal.

BSA and fibrinogen, respectively. As described above, the tilt of the liquid crystals was 1.5 ± 0.5° prior to immersion of the rubbed films of BSA into aqueous solutions of BSA or fibrinogen. This result suggests that nonspecific adsorption gives rise to a small change (2° or less) in the tilt of the liquid crystals. We conclude, therefore, that rubbed films of covalently immobilized BSA limit the nonspecific adsorption of proteins to levels that largely sustain the uniform planar orientation of 5CB in a direction that is parallel to the direction of rubbing of the BSA.

Orientation of 5CB Following Specific Binding of Anti-BSA IgG to Rubbed Films of BSA. The results above establish that rubbed films of covalently immobilized BSA can uniformly orient liquid crystals before and after immersion into aqueous solutions of proteins that do not have specific interactions with

Table 3. Fraction of Light Transmitted through Liquid Crystals Anchored on Rubbed Films of Covalently Immobilized BSA

protein (concentration)	fractional transmittance		
	I_{Max}[b]	I_{Min}[c]	$(I_{max} - I_{Min})/I_{Max}$[d]
reference[e,f]	0.63 ± 0.02	0.03 ± 0.01	0.94 ± 0.01
	0.55 ± 0.01	0.04 ± 0.01	0.93 ± 0.01
BSA (10 mg/mL)[f]	0.61 ± 0.02	0.05 ± 0.01	0.91 ± 0.02
	0.53 ± 0.04	0.03 ± 0.01	0.95 ± 0.01
fibrinogen (0.2 mg/mL)[f]	0.58 ± 0.05	0.09 ± 0.02	0.84 ± 0.02
	0.53 ± 0.04	0.07 ± 0.02	0.83 ± 0.04
lysozyme (0.2 mg/mL)	0.49 ± 0.02	0.02 ± 0.01	0.94 ± 0.01
anti-BSA IgG (100 nM)[f,g]	0.34 ± 0.04	0.23 ± 0.02	0.33 ± 0.04
	0.27 ± 0.02	0.19 ± 0.04	0.32 ± 0.08
anti-FITC IgG (100 nM)[g]	0.58 ± 0.06	0.01 ± 0.01	0.96 ± 0.01
anti-streptavidin IgG (100 nM)[g]	0.57 ± 0.01	0.03 ± 0.01	0.94 ± 0.01

[a] Fractional transmittance was measured between crossed polarizers after immersion of the rubbed BSA films into protein solutions for 2 h. The uncertainty in each measurement is based on the sampling of 3–5 fields of view of each cell. [b] The maximum values (I_{Max}) of fractional transmittance were measured when the angle between the polarizer and the rubbing direction of the optical cell was 45, 135, 225, and 315°. [c] The minimum values (I_{Min}) were obtained when the angle between the polarizer and the rubbing direction of the optical cell was 0, 90, 180, and 270°. [d] The values of $[I_{Max} - I_{Min}]/I_{Max}$ were calculated from the paired fractional transmittances at (0°, 45°), (90°, 135°), (180°, 225°), and (270°, 315°). [e] The reference corresponds to a rubbed film of immobilized BSA not subsequently immersed into a protein solution. [f] The transmittances for two cells were measured in order to observe the sample-to-sample variation. [g] 100 nM of IgG corresponds to 0.015 mg/mL.

the BSA. Here we report the specific binding of a protein to a rubbed film of BSA and the consequence of the binding on the orientations of liquid crystals. We immersed a rubbed film of covalently immobilized BSA into a solution of aqueous PBS containing 100 nM of IgG for 2 h. Following the withdrawal of the rubbed film of BSA from an anti-BSA IgG solution, we observed the optical appearance of the liquid crystal to be highly nonuniform (Figure 6D). Rotation of the sample between crossed polarizers did not result in reproducible modulation in the intensity of light transmitted through the sample (Figure 7). In contrast, following the withdrawal of rubbed films of BSA from aqueous solutions containing nonspecific IgGs (anti-FITC IgG and anti-streptavidin IgG), we measured the orientation of the liquid crystal to be uniform and planar (Figure 6E and F). We conclude, therefore, that specific binding of the anti-BSA IgG erased the anisotropic structure of the rubbed film of BSA responsible for the uniform alignment of liquid crystals.

We also performed ellipsometric measurements of the extent of binding of IgGs to rubbed films of BSA (Table 2). We measured the extent of specific binding of anti-BSA IgG on both rubbed and unrubbed films of BSA to be similar and large (~40 Å). In contrast, the level of adsorption of the nonspecific IgGs was measured to be small (~5 Å). These measurements of ellipsometric thicknesses support our conclusion that specific binding of anti-BSA to the rubbed film of BSA erases the anisotropic structure of the BSA film that was induced by rubbing and thus leads to nonuniform alignment of the liquid crystals.

Quantitative Analysis of the Optical Appearance of 5CB Induced by Specific and Nonspecific Binding of Protein. We quantified the optical appearance of liquid crystals supported on rubbed films of BSA before and after specific and nonspecific binding of proteins by measurement of the modulation in the intensity of light transmitted through the optical cell during its rotation between crossed polarizers. Figure 7 reveals distinct differences in the optical response of the liquid crystals induced by specific binding of anti-BSA and nonspecific binding events. In particular, we point out that the rubbed film of BSA immersed into an aqueous solution of fibrinogen, while inducing visible defects in the liquid crystals (Figure 6C), possesses an optical response that is clearly distinguishable from the response of the film of BSA on which anti-BSA IgG was specifically bound. Table 3 summarizes the results shown in Figure 7 in terms of a numerical index $([I_{Max} - I_{Min}]/I_{Max})$ calculated from the maximum (I_{Max}) and minimum (I_{Min}) values of the fractional transmittance during rotation of the sample between crossed polarizers. The value of $[I_{Max} - I_{Min}]/I_{Max}$ corresponding to specific binding of anti-BSA IgG was ~0.33, whereas all other samples (nonspecific adsorption) possessed values of the index that were greater than 0.84. In particular, we point out that the value of $[I_{Max} - I_{Min}]/I_{Max}$ for fibrinogen (~0.84) is much closer to the value measured on the rubbed film of BSA not subsequently immersion into a protein solution (~0.94) than rubbed BSA to which anti-BSA IgG was specifically bound (~0.33).

CONCLUSIONS

We conclude that rubbed films of BSA covalently immobilized on the surface of glass microscope slides can orient liquid crystals uniformly, resist nonspecific adsorption of proteins from aqueous solutions at levels that are sufficient to maintain uniform alignment of liquid crystals, and yet possess an anisotropic structure that can be erased by specific binding of proteins to these surfaces. These properties, when combined, suggested that rubbed films of proteins may be useful as substrates on which specific biomolecular interactions can be imaged by using liquid crystals.

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